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Cloning and Characterization of the Xyn11A Gene from *Lentinula edodes*

Charles C. Lee,[1,2] Dominic W. S. Wong,[1] and George H. Robertson[1]

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Hemicellulose represents a rich source of biomass that can be converted into useful chemical feedstocks. One of the main components of hemicellulose is xylan, a polymer of xylose residues. Xylanase enzymes that hydrolyze xylan are therefore of great commercial interest. We have cloned a gene (*xyn11A*) that encodes a 283-amino acid xylanase enzyme from the fungus *Lentinula edodes*. The enzyme has a pI of 4.6 and belongs to the highly conserved glycosyl hydrolase family 11. The xylanase gene was cloned into a *Pichia pastoris* expression vector that secretes active enzyme into both solid and liquid media. The optimal reaction conditions were at pH 4.5 and 50°C. The enzyme had a K_m of 1.5 mg/ml and a V_{max} of 2.1 mmol/min/mg. Xyn11A produced primarily xylobiose, xylotriose, and xylotetraose from a birchwood xylan substrate. This is the first report on the cloning of a hemicellulase gene from *L. edodes*.

KEY WORDS: hemicellulose; *Lentinula edodes*; xylanase.

1. INTRODUCTION

Hemicellulose is second only to cellulose as a main component of lignocellulose plant biomass (Ward and Moo-Young, 1989). The main portion of hemicellulose is xylan which is a polymer consisting primarily of beta-1,4-linked xylose residues. Because the chemical constituents of lignocellulose represent the main feedstock alternative to petroleum-based products, there is great interest in improving the efficiency by which the lignocellulose can be hydrolyzed to simpler compounds. In nature, the beta-1,4 bonds between the xylose residues are digested by xylanase enzymes (Prade, 1996). There are several general categories of such enzymes. The endo-xylanases digest the internal beta-1,4 bonds. Beta-xylosidases digest the xylosidic bonds from the ends of the xylan polymer. Some beta-xylosidases also function as xylobiases. In addition, there are an assortment of other enzymes that remove various chemical side chains that are attached to the main xylan polymer (Biely *et al*., 1986; Bajpai, 1997). All of these enzymes work in concert to synergistically hydrolyze xylan.

The xylanase enzymes can be used in conjunction with cellulase enzymes to hydrolyze the lignocellulose substrate into sugars that can then be fermented by microorganisms into products such as ethanol and lactic acid (Chandrakant and Bisaria, 1998). The xylanase enzymes can also be used alone to produce purer cellulose preparations (Beg *et al*., 2001). For instance, xylan can be removed enzymatically during the paper pulping process thus decreasing the chemical usage and costs normally associated with this procedure.

In an effort to improve the efficiency of xylan degradation for biofuel ethanol production, we are

[1] Western Regional Research Center, USDA-ARS, Albany, CA, U.S.A.
[2] To whom correspondence should be addressed. USDA-ARS, 800 Buchanan St., Albany, CA 94710. Fax: + 1-510-559-5940. E-mail: clee@pw.usda.gov

[3] Abbreviations: Asp, aspartic acid; Avi, avicel; BeX, beechwood xylan; BiX, birchwood xylan; CMC, carboxymethyl cellulose; DNSA, dinitrosalicyclic acid; OSX, oat spelt xylan; PCR, polymerase chain reaction; RACE-PCR, rapid amplification of cDNA ends; RBB, remazol brilliant blue; RFU, relative fluroscence units; VAl, valine; Xyn, xylanase; YEPD, yeast extract-peptone-dextrose growth media.

isolating novel xylanase genes as a first step in engineering improved activities into these enzymes. We are cloning xylanase genes from organisms that would be expected to encode such enzymes. We have isolated a gene, *xyn11A*, encoding a xylanase enzyme from *Lentinula edodes*, a fungus with high commercial value as a food, Shiitake mushroom. *L. edodes* is traditionally grown on fallen logs and secretes a variety of lignocellulolytic enzymes (Leatham, 1985). The predicted Xyn11A amino acid sequence identifies the enzyme as a family 11 glycosyl hydrolase (Henrissat and Bairoch, 1996). Active Xyn11A enzyme was successfully expressed from a *Pichia pastoris* expression vector. The enzyme activity was determined to be optimal at 50°C and pH 4.5. Xyn11A released primarily xylobiose, xylotriose, and xylotetraose from a birchwood xylan substrate. Kinetic analyses demonstrated that the K_m was 1.5 mg/ml and V_{max} was 2.1 mmol/min/mg.

2. MATERIALS AND METHODS

2.1. Fungus and Growth Conditions

Lentinula edodes strain Stamets CS-2 (Fungi Perfecti, Olympia, WA) was cultured at room temperature on alder sawdust blocks. Mycelium was harvested with a scalpel at various stages (pinning, button, and veil break) of mushroom development. The mycelium samples were pooled and frozen in liquid nitrogen and stored at −80°C until further processing.

2.2. RNA Isolation

Total RNA was collected from mycelium grown on alder sawdust. The mycelium was harvested and then ground to a fine powder in liquid nitrogen with a mortar and pestle. 100 mg of tissue powder was then lysed and total RNA was collected with the RNeasy Plant Kit (Qiagen, Valencia, CA). The RNA was treated with DNaseI and again purified with the RNeasy Plant Kit. Then, the Oligotex mRNA kit (Qiagen) was used to purify poly-A mRNA from the total RNA.

2.3. Cloning Xylanase Gene

The strategy for cloning the xylanase gene has been previously described (Lee *et al.*, 2001). The mRNA isolated from the sawdust-grown mycelium was used as the starting material to isolate the xylanase gene. The GeneRacer kit (Invitrogen, Carlsbad, CA) was used to conduct Rapid Amplification of cDNA Ends-PCR (RACE-PCR). In brief, the mRNA was treated with calf intestinal phosphatase followed by tobacco acid pyrophosphatase to remove the cap structure. An RNA oligonucleotide linker was ligated to the 5′ end of the decapped mRNA. cDNA was then generated by reverse-transcribing the mRNA with a poly-dT oligonucleotide linker localized to the 3′ end.

cDNA resulting from 6.25 ng of mRNA served as a template in PCR reactions using both a primer directed against the known 5′ or 3′ linker ends of the mRNA and degenerate primers that were designed based upon known carbohydrate-binding domain sequences from cloned genes encoding fungal enzymes. PCR was carried out with PfuTurbo Hotstart DNA polymerase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Products of the PCR reaction were cloned into pCR4Blunt-TOPO vector (Invitrogen) and sequenced. This sequencing information was then used to design another set of internal primers against the putative xylanase gene. These new primers were used in conjunction with the 5′ and 3′ primers (directed against the known linker regions) in another round of PCR using the cDNA as a template. These final PCR reactions produced the 5′ and 3′ halves of the xylanase gene that were then assembled into full-length constructs in the pCR4Blunt-TOPO vector.

2.4. Xylanase Expression and Purification

To express active xylanase, the *xyn11A* gene fused to a 6X His-tag at the C-terminus was subcloned into the pGAPZαA plasmid (Invitrogen). The expression vector was digested with *Avr*II restriction enzyme (New England Biolabs, Beverly, MA) and transformed into the *P. pastoris* strain GS115, and antibiotic-resistant colonies were selected on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plates containing 1 *M* sorbitol and 100 μg/ml zeocin. Liquid YPD cultures were inoculated with the transformed GS115 cells and shaken at 225 rpm at 30°C. After 72 h, the culture was spun down to remove the cells, and the supernatant containing the secreted enzyme was collected.

The enzyme was bound to a HiTrap Chelating HP-nickel column (Amersham Biosciences, Piscataway, NJ) and eluted with buffer containing 20 m*M* sodium phosphate (pH 7.4), 0.5 *M* sodium chloride, and varying amounts of imidazole. The fractions were analyzed on an SDS-PAGE gel, and those containing pure protein were pooled. The purified protein was then applied to the HiTrap Desalting column (Amersham Biosciences) that had been equilibrated with 25 m*M* sodium acetate (pH 4.5).

2.5. Xylanase Activity Assays

To assay activity on a solid substrate, GS115 colonies carrying the Xyn11A expression vectors were spotted onto YPD agar plates containing 0.2% wheat arabinoxylan dyed with remazol brilliant blue (RBB-xylan) (Megazyme, Bray, Ireland). Activity was indicated by the formation of clear halos around the individual clones.

pH and temperature optimums were determined using RBB-xylan as substrate in liquid activity assays. To measure the pH optimum, 50 µl of an RBB-xylan solution (10 mg/ml) was added to 100 µl of solution containing 50 n*M* xylanase enzyme and 25 m*M* of buffer ranging from pH 2.5–3.5 (glycine), pH 4.0–5.5 (sodium acetate), and pH 6.0–8.0 (sodium phosphate). After 30 min at 30°C, the reaction was stopped by the addition of 2X volume of 95% ethanol. The samples were spun down, and the resulting supernatant containing the soluble, digested xylan fraction was measured on a 96-well microplate reader at a wavelength of 620 nm. The temperature optimum assays were conducted in a similar fashion except pH 4.5 was constantly maintained.

To determine the kinetic parameters, birchwood xylan (Sigma, St. Louis, MO) was used as the substrate. Reactions were conducted at 50°C in 100 µl containing 25 m*M* sodium acetate (pH 4.5), 25 nM of Xyn11A enzyme, and birchwood xylan (1 to 9 mg/ml). The reactions were stopped by the addition of 150 µl of dinitrosalicyclic acid solution (0.1 g DNSA dissolved in 8 ml 0.5 *M* sodium hydroxide, followed by 3 g of potassium sodium tartrate, with the final volume raised to 10 ml by the addition of 0.5 *M* sodium hydroxide) and heating the sample to 100°C for 5 min. The samples were then measured on a 96-well plate reader at 562 nm wavelength. Maltose solutions were used as standards to calibrate the signal to amount of product.

2.6. Analysis of Xyn11A Reaction Products

Xyn11A reaction products were analyzed on an eCAP N-CHO coated capillary installed on the P/ACE MDQ Capillary Electrophoresis System with a 488 nm laser module (Beckman Coulter, Fullerton, CA). First, 140 ng of Xyn11A was incubated in 25 µl of a 1% birchwood xylan solution (pH 4.5) at 50°C for 16 h. The reaction was stopped by adding 3 volumes of −20°C ethanol and placed on ice for 10 min. The tubes were spun 5 min at 4°C to pellet the protein. The supernatant containing the reaction products was transferred to a new tube and dried in a centrifugal vacuum evaporator. The reducing sugar ends in the resulting pellet were labeled with a fluorophore from the Carbohydrate Labeling and Analysis Kit (Beckman Coulter) according to manufacturer's protocol. The reaction products were applied to the coated capillary and migration was visualized by laser induced fluorescence. The xylooligomer standards were used to identify the peaks.

3. RESULTS AND DISCUSSION

3.1. Cloning of *Xyn11A* Gene

We cloned the Xyn11A gene from the fungus *L. edodes* with a RACE-PCR strategy by employing degenerate primers directed against the conserved carbohydrate-binding domain (Tilbeurgh *et al.*, 1986; Johansson *et al.*, 1989) (see Materials and methods). *Xyn11A* was a 849-base pair gene that encoded a 283-amino acid protein with a calculated molecular weight of 29.5 kD and a pI of 4.6 (Fig. 1). As would be expected of an enzyme that is secreted, there was a signal peptide at the N-terminus that was predicted to be cleaved between Asp20 and Val21 (Nielsen *et al.*, 1997). The Xyn11A enzyme had a 178-amino acid catalytic domain (residues 41–219) that categorized the protein as a member of the glycosyl hydrolase family 11 (Henrissat and Bairoch, 1996). A serine/threonine-rich linker sequence (residues 223–248) connected the catalytic domain to a 33-amino acid fungal-type carbohydrate-binding domain (residues 251–283) at the C-terminus of the protein. The Xyn11A enzyme also contained two conserved residues (Glu116 and Glu207) that were implicated as the catalytic acid-base pair based on detailed studies of other family 11 xylanases (Ko *et al.*, 1992; Miao *et al.*, 1994; Wakarchuk *et al.*, 1994). When compared to other

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ATG GCC TAC AAG TCT TTA CTT TTC CTC GCT TTG ATC GCG GTT ACC GCT ACT GCC CTC GAT GTC TTT GAC AAC TCG
 M  A  Y  K  S  L  L  F  L  A  L  I  A  V  T  A  T  A  L  D  V  F  D  N  S   25

ACT GAG GTC ATA GGC AAA CGA AGT ATC CCG AAC GGA GAA GGA ACC AAT AAT GGC TAC TTC TAC TCA GTT TAT TCC
 T  E  V  I  G  K  R  S  I  P  N  G  E  G  T  N  N  G  Y  F  Y  S  V  Y  S   50

GAT ACC ACC GTT ACA GGG ACT TAC ACG AAT GGT CCA GGT GGA GAA TAC ACC CTT ACC TGG GGC GGA TCA GGA GAC
 D  T  T  V  T  G  T  Y  T  N  G  P  G  G  E  Y  T  L  T  W  G  G  S  G  D   75

GTC GTA GTA GGG AAG GGA TGG AAC CCA GGA GGC CCG ATG TCT GTC GAG TAC AGT GGT ACT TAC TCC CCC AAC GGA
 V  V  V  G  K  G  W  N  P  G  G  P  M  S  V  E  Y  S  G  T  Y  S  P  N  G  100

AAC TCG TAT CTT TCA GTG TAC GGC TGG ATG ACG AGT CCC CTT GTT GAG TAT TAC ATT ACT GAC TCT TTC GGT GAT
 N  S  Y  L  S  V  Y  G  W  M  T  S  P  L  V  E  Y  Y  I  T  D  S  F  G  D  125

TAC AAT CCC AGC ACT GGC GGA ACT CAC CTG GGG ACT TGC ACA AGT GAC GGA GGA GTC TAC GAT ATA TAC ACC CAA
 Y  N  P  S  T  G  G  T  H  L  G  T  C  T  S  D  G  G  V  Y  D  I  Y  T  Q  150

ACC CGC ACG AAT GCG CCG TCA ATT CAA GGG ACT GCC ACA TTC CAG CAG TAC TGG TCC ATC CGC CAA ACT CAT CGG
 T  R  T  N  A  P  S  I  Q  G  T  A  T  F  Q  Q  Y  W  S  I  R  Q  T  H  R  175

GTC GGT GGC ACC GTC ACC ACG GGC AAC CAC TAC TCC TGC TGG GAG TCA GTC GGT TTG CCT CTA GGC ACG TTC AAC
 V  G  G  T  V  T  T  G  N  H  Y  S  C  W  E  S  V  G  L  P  L  G  T  F  N  200

TAC ATT ATC CTC GCG ACC GAA GGA TAC TCT TCA AGC GGG ACC TCC ACT ATC ACG GTC GGC CAA GGC ACT GGA ACA
 Y  I  I  L  A  T  E  G  Y  S  S  S  G  T  S  T  I  T  V  G  Q  G  T  G  T  225

GGT TCA TCA GCT CCT TCT GGG CCT TCT TCA ACG ACT ACT ACC CCT CCG ACT GCT CCT ACA GGA GGA ACA GTC GCT
 G  S  S  A  P  S  G  P  S  S  T  T  T  P  P  T  A  P  T  G  G  T  V  A  250

CAG TGG GGT CAA TGT GGT GGC ATA GGG TAT TCC GGC CCG ACA ACT TGC GCT TCT CCG TAT ACA TGC ACT GTT GCC
 Q  W  G  Q  C  G  G  I  G  Y  S  G  P  T  T  C  A  S  P  Y  T  C  T  V  A   275

AAC GCG TAC TAT TCT CAG TGT CTC TAA
 N  A  Y  Y  S  Q  C  L  *  283
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Fig. 1. *Xyn11A* gene and predicted amino acid sequence. Highlighted residues denote the signal peptide. Underlined sequences mark the carbohydrate-binding motif. GenBank accession number AF411252.

family 11 fungal xylanases, the Xyn11A enzyme had high sequence similarities to enzymes that had been classified as endo-xylanases (Fig. 2). For example, there is greater than 60% identity between the catalytic domains of the *L. edodes* Xyn11A and the *P. chrysosporium* XynB enzymes.

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L.e.-XYN11A    1 MA-YKSLLFLALIAVTATALDVFDNSTEVIGKR----------S-IPNGEGTNNG
P.c.-XYNB      1 MVSFNSLLVAVSAATCALAFPFEFHNGTHVFFR----------QSTPAGTGTNNG
F.o.-XYL5      1 MVHFTSVFAGLSLVAGSLAAPSKEGLFSKITKR----------AGTPNSSGTNNG
T.r.-XYN1      1 MVSFTSLLAASPPSR-ASCRPAAEVESVAVEKR----------QTIQPGTGYNNG
A.o.-XYNG2     1 MVSFSSILLACSAAIGALATPIEPLADHPNEAFNETAFNDLVGRSTPSSTGYNNG

L.e.-XYN11A   44 YFYSVYSDTTVTGTYTNGPGGEYTLTWGGSGDVVVGKGWNPG-GPMSVEYSGTYS
P.c.-XYNB     46 YFYSFWTDGGGSVTYNNGPAGEYSVTWSNADNFVAGKGWNPG-SAQAISFTANYQ
F.o.-XYL5     46 FYYSWWSDGGADATYTNGEGGSYSMEWKDGGNVVGGKGWSPG-KARTISYEGEYK
T.r.-XYN1     45 YFYSYWNDGHGGVTYTNGPGGQFSVNWSNSGNFVGGKGWQPGTKNKVINFSGSYN
A.o.-XYNG2    56 YYYSFWTDGGGDVTYTNGNGGSYSVQWSNVGNFVGGKGWNPG-SSRAITYSGSFN

L.e.-XYN11A   98 PNGNSYLSVYGWMTSPLVEYYITDSFGDYNPSTGGTHLGTCTSDGGVYDIYTQTR
P.c.-XYNB    100 PNGNSYLSVYGWSTNPLVEYYILPDFGTYNPAVSLTHKGTLTSDGATYDVYEGTR
F.o.-XYL5    100 PNGNSYLSVYGWTRNPLVEYYIVESFGTYNPSSGATKKGTVEADGSTYDIFETTR
T.r.-XYN1    110 PNGNSYLSVYGWSRNPLIEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQR
A.o.-XYNG2   111 PSGNGYLAVYGWTTDPLIEYYIVESYGTYNPGSGGTYKGQVTSDGSTYNIYTSVR

L.e.-XYN11A 153 TNAPSIQGTATFQQYWSIRQTHRVGGTVTTGNHYSCWESVGLPLGTFNYIILATE
P.c.-XYNB   155 VNEPSIQGTATFNQYWSIRSSKRSSGTVTTANHFAAWKQLGLPLGTFNYQIVATE
F.o.-XYL5   155 TNAPSIDGTQTFQQYWSVRQQHRSTGSVDTGLHFDAWEKAGMKLGTHDYQILATE
T.r.-XYN1   155 VNQPSIIGTATFYQYWSVRRNHRSSGSVNTANHFNAWAQQGLTLGTMDYQIVAVE
A.o.-XYNG2  165 TNAPSIIGTATFTQFWSVRTSKRVGGTVTTGNHFNAWAKYGLTLGTHNYQIVATE

L.e.-XYN11A 208 GYSSSGTSTITVG-QGTGTGSSAPSGPSSTTTTPP-----TAPTGGTVAQWGQCG
P.c.-XYNB   210 GYQSSGSSTVTVNPAGGVTSPTAPTGPSSVSTTPSGPSSSPSPVGSCAALYGQCG
F.o.-XYL5   210 GYFSSGSSHMTVSEGASSGGGAGGSTGGDASQGGDSQQGGDVQQGGDASQGGNGQ
T.r.-XYN1   210 GYFSSGSASITVS-----------------------------------------
A.o.-XYNG2  220 GYQSSGSSAITVY-----------------------------------------

L.e.-XYN11A 257 ----GIGYSGPTTCASPYTCTVANAYYSQCL
P.c.-XYNB   265 ----GQGWTGPTCCSS-GTCKFSNNWYSQCL
F.o.-XYL5   265 QGGNGNSFQQPGSENQPQQQEIDTGANEPCQ
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Fig. 2. Comparison of Xyn11A to other fungal xylanases. Stars above residues indicate the conserved catalytic amino acids. Abbreviations: L.e., *Lentinula edodes*; P.c., *Phanerochaete chrysosporium*; F.o., *Fusarium oxysporum* (Gomez-Gomez *et al*., 2001); T.r., *Trichoderma reesei* (Torronen *et al*., 1992); and A.o., *Aspergillus oryzae* (Kimura *et al*., 2000).

3.2. Activity of Xylanase Enzyme

In order to facilitate future genetic engineering of the Xyn11A enzyme, an expression system was required that would allow for rapid screening of active enzyme on solid and liquid growth media. Attempts to produce Xyn11A enzyme in *Saccharomyces cerevisiae* and *Escherichia coli* resulted in protein that was not active (data not shown). The *xyn11A* gene was then subcloned into the plasmid pGAPZαA that was then transformed into the *P. pastoris* strain GS115. This expression vector directs the secretion of heterologous proteins into the growth media. Individual GS115 clones transformed with the pGAPZαA expression vector with or without the *xyn11A* gene were spotted onto YPD plates containing 0.2% mg/ml RBB-xylan, a dye-labeled substrate (Fig. 3). After growth in a 30°C incubator, colonies transformed with the pGAPZαA vector carrying the *xyn11A* gene secreted active xylanase enzyme into the solid media containing soluble xylan substrate. This activity was visualized by the formation of clear halos around individual colonies.

The transformed GS115 clones also secreted active xylanase enzyme into liquid culture media. Cultures were inoculated into YEPD culture media and shaken at 30°C for 72 h. The secreted xylanase was purified and used to biochemically characterize the enzyme activity. The optimal pH and temperature were demonstrated to be pH 4.5 and 50°C, respectively (Fig. 4). Kinetic experiments using birchwood xylan as substrate demonstrated that the K_m was 1.5 mg/ml and the V_{max} was 2.1 mmol/min/mg.

We tested the activity of Xyn11A against various substrates (Fig. 5). The enzyme had the most activity against xylan from beechwood and slightly lower activities against xylan from birchwood and oat spelts. No significant activity was detectable against the substrates carboxymethyl cellulose and Avicel.

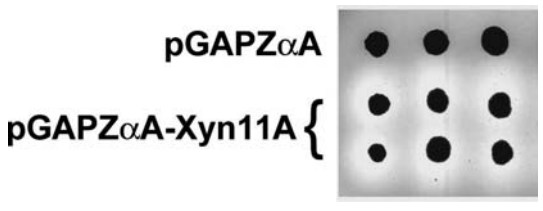

Fig. 3. Xylanase digestion on solid media. *P. pastoris* GS115 clones transformed with either pGAPZαA with or without the *Xyn11A* gene were spotted onto YPD containing 0.2% RBB-xylan.

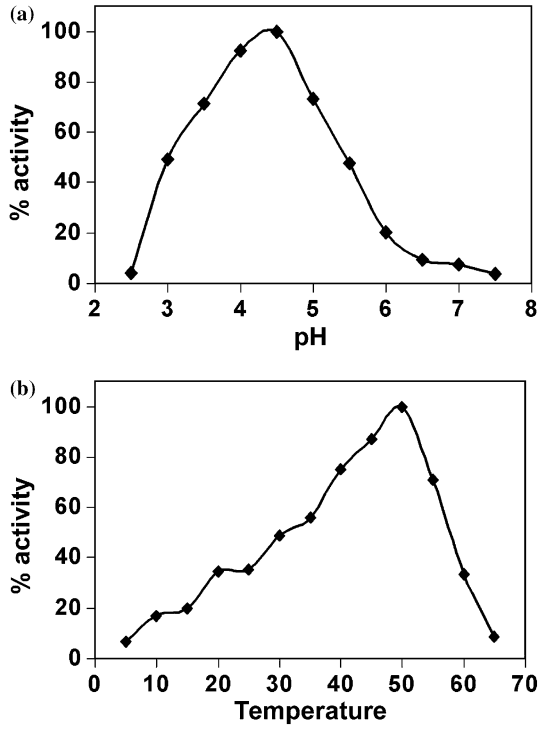

Fig. 4. pH and temperature activity profile of Xyn11A enzyme. RBB-xylan was incubated with Xyn11A enzyme at (a) varying pH's and (b) various temperatures to determine the optimum reaction conditions.

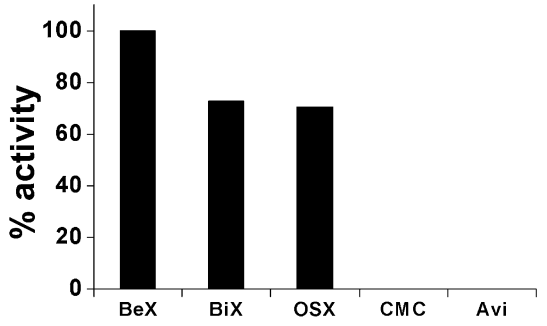

Fig 5. Substrate susceptibility. Various substrates were incubated with Xyn11A at 50°C and pH 4.5 for 10 min. The reactions were stopped with DNSA solution and analyzed in a plate reader (see Materials and methods). BeX, beechwood xylan; BiX, birchwood xylan; OSX, oat spelt xylan; CMC, carboxymethyl cellulose; and Avi, avicel.

To determine the digest products of the Xyn11A enzyme, we analyzed the reactions on a capillary electrophoresis column. The enzyme releases primarily xylobiose, xylotriose, and xylotetraose as reaction products, but no xylose (Fig. 6). These data demonstrate the Xyn11A enzyme is an endoxylanase.

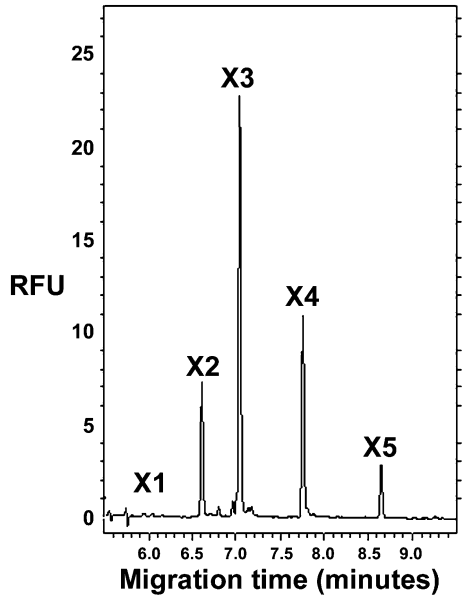

Fig. 6. Xyn11A reaction products. The xylanase enzyme was incubated with birchwood xylan and the reaction products were analyzed on a capillary electrophoresis column. RFU, relative fluorescence units; X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; and X5, xylopentaose.

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